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# PROPERTIES OF ERYTHROCYTES WHICH MAY INFLUENCE THE CONVERSION OF PROFIBRINOLYSIN TO FIBRINOLYSIN

SCHOOL OF AVIATION MEDICINE
RANDOLPH AIR FORCE BASE, TEXAS

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# PROPERTIES OF ERYTHROCYTES WHICH MAY INFLUENCE THE CONVERSION OF PROFIBRINOLYSIN TO FIBRINOLYSIN

S. N. KOLMEN, Ph.D.
M. MASON GUEST, Ph.D.
D. R. CELANDER, Ph.D.

Carter Physiology Laboratory and Department of Biochemistry
University of Texas Medical Branch
Galveston, Texas

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Air University
SCHOOL OF AVIATION MEDICINE, USAF
RANDOLPH AFB, TEXAS
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# PROPERTIES OF ERYTHROCYTES WHICH MAY INFLUENCE THE CONVERSION OF PROFIBRINOLYSIN TO FIBRINOLYSIN

Evidence is presented that erythrocytes adsorb urokinase, a urinary activator of plasma profibrinolysin, and that in the adsorbed state urokinase not only remains active but is "protected" from factors normally present in plasma. When washed red cells, devoid of components of the fibrinolytic system, are exposed to plasma they acquire simultaneously inhibitors to urokinase and an agent capable of lysing a fibrin clot. Evidence is presented that these acquired properties are the result of adsorption to the erythrocyte of factors present in plasma and that this adsorption is affected by the fibrinolytic status of the animal from which the plasma and erythrocytes are obtained. The significance of the erythrocyte in the development of in vivo fibrinolytic activity is discussed.

Physiologic activation of the fibrinolytic enzyme system results in the dissolution of clots formed in the body. Previous studies (1, 2, 3) indicate that this dissolution is a consequence of factors present within the confines of the clot itself. The presence of the fibrinolytic factors in a forming clot may be the result of sequestration of formed elements by the fibrin network. If this postulate is to be tested, information concerning participation by the various components making up a clot is needed. Thus far, components of the in vivo system such as plasma (4), platelets (5), red cell stroma, and white cells (6) have been reported to contain factors which influence the activation of the fibrinolytic system. An agent which has been neglected, although it is enmeshed in a fibrin clot in vivo and is thus potentially a factor in influencing the fate of the clot, is the erythrocyte.

This report presents evidence that erythrocytes, through the adsorption of one or more fibrinolytic components involved in the conversion of profibrinolysin to fibrinolysin, may be more closely associated with the in vivo activation of the fibrinolytic enzyme system than has been generally appreciated.

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## MATERIAL AND METHODS

Sheep erythrocytes were obtained from the Brown Laboratory of Topeka, Kans. These cells were usually used within two weeks and never more than six weeks after being removed from the animal source. The cells were washed at least 3 times in veronal-saline buffer, pH 7.4, before being used. Erythrocyte stroma were obtained through hypotonic hemolysis of the cells.

Urokinase, an agent which is found in the urine of a number of species (7) and which acts directly in the conversion of plasma profibrinolysin to fibrinolysin, was isolated, purified, and assayed by methods developed in this laboratory (8, 9). Such preparations were diluted in saline to the unitages specified. The unit has been defined elsewhere (9).

A frozen stock solution of bovine fibrinogen, isolated by the freeze-thaw technic of Ware et al. (10), was thawed and then diluted to a final concentration of 0.2 percent clottable protein (3).

Topical thrombin, in vials containing approximately 5,000 NIH units, was dissolved in 2.5 ml. of 0.9 percent saline and 1 ml. of glycerol to form a solution containing approximately 1,400 NIH units per milliliter. This was stored at 4° C.

Purified beef lung thromboplastin was prepared as described elsewhere (11). Frank fibrinolytic activity was determined by the tilt-tube method described by Guest (12).

## EXPERIMENTAL AND RESULTS

Preliminary tests indicated that when washed erythrocytes were suspended in a urokinase solution, a portion of the kinase activity which invariably disappeared from the solution appeared on the washed red cells. To gain some idea of the quantitative relationships involved, 2.5 ml. of 20 percent sheep red cells were mixed with 2.5 ml. of urokinase solution containing 70 centiunits of activity. This mixture was incubated at 37° C. for 15 minutes before the cells were sedimented at low speed (5,000 relative centrifugal force for 10 minutes at 0° C.) Assays for lytic activity were performed on the supernatant solution. The cells were washed once with veronal-saline buffer, pH 7.4 suspended in 9 times their volume of 1 M KSCN solution for 30 minutes at 27° C., and separated from the KSCN eluate by centrifugation at low speed. The KSCN elution procedure was repeated twice. Aliquots of each eluate were dialyzed against frequent changes of distilled water at 4° C. until they no longer gave a positive test for thiocyanate ion when tested with 1 percent ferric chloride solution. Aliquots of erythrocytes which had been suspended in urokinase solution and of the dialyzed KSCN eluates were assayed for lytic activity. The results are summarized in table I. Fifty-five percent of the urokinase which disappeared from the solution as a result of exposure to erythrocytes was recovered by KSCN elution of the red cells. Eluates obtained by KSCN treatment of erythrocytes unexposed to urokinase contained *neither kinase nor fibrinolytic activity.*

To determine whether the property of urokinase adsorption is unique for intact erythrocytes the experiment was repeated, substituting red cell stroma for the intact cells. Two mg. of erythrocyte stroma were mixed with 1 ml. of solution containing 147 centiunits of urokinase activity. After incubation in the same manner as for intact red cells the stroma was sedimented at high speed (23,000 relative centrifugal force for 10 minutes at 0° C.). The sedimented residue was washed with veronal-saline buffer, pH 7.4, and extracted twice with 1 M KSCN. The supernatant solution separated from the stroma, the washed stroma, and the two KSCN extracts were assayed for urokinase content. From the data presented in table I it is apparent that the kinase activity which disappeared from solution when in contact with red cell stroma was recovered upon elution of the stroma. In similar experiments, lyophilized stroma from the same red cell source did not bring about a decrease in activity in the urokinase solution and such preparations did not exhibit activity after exposure to urokinase. When canine or human erythrocytes were substituted for sheep erythrocytes in these experiments, results similar to those shown in table I were obtained.

TABLE I

*Distribution of urokinase between aqueous and solid phase*

| | Red cells | Stroma | Lyophilized stroma |
|---|---|---|---|
| Kinase initially present | 0 | 0 | 0 |
| Urokinase added (centiunits) | 70 | 147 | 147 |
| Urokinase remaining in solution after exposure to solid phase (percent) | 69 | 91 | 100 |
| Urokinase removed by solid phase (percent) | 31 | 9 | 0 |
| Solid phase activity removed on KSCN elution (percent) | 55 | 100 | — |

The apparent adsorption of urokinase activity on red cells was found to be influenced by pH. Buffers of ionic strength, 0.1, ranging from pH 5.0 through 9.0, were used to prepare suspensions of washed sheep erythrocytes with a final cell concentration of 20 percent by volume. One-ml. aliquots of these buffered cell suspensions were mixed with an equal volume of urokinase solution containing 110 centiunits of activity per milliliter. After incubation for 30 minutes at 37° C. the mixtures were sedimented at low speed. The erythrocytes, after being washed once, and the supernatant solutions were assayed for urokinase activity. Control systems consisting of buffers with erythrocytes in the absence of urokinase, buffers plus urokinase, and buffers alone were also assayed for kinase activity. The data, summarized in figure 1, show that, while little alteration in urokinase activity occurred at the various pH levels, the pH markedly influenced the distribution of urokinase between the cells and the solution, kinase being most strongly associated with the cells at pH 8.0.

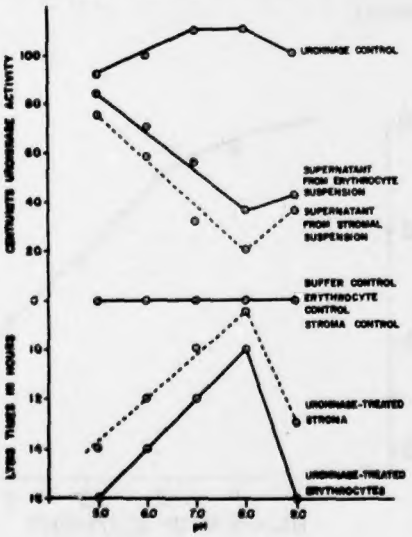


**FIGURE 1**

*Effect of pH on adsorption of urokinase by erythrocytes.*

Similar results were obtained in experiments in which 2 mg. of stroma were substituted for the intact red cells. As illustrated in figure 1 the affinity of urokinase for stroma was also greatest at pH 8.0. Stroma without added urokinase exhibited no kinase activity when incubated at pH 5.0 through 9.0.

To gain information about the mode of binding of urokinase to erythrocytes and erythrocyte stroma, experiments were performed in which a constant concentration of erythrocytes, at a constant temperature, was exposed to varying amounts of urokinase. Suspensions having a total volume of 1 ml. were formed by mixing equal volumes of 20 percent erythrocytes and veronal-saline buffered solutions, pH 7.4, containing 4 to 150 centiunits of urokinase activity. All suspensions were incubated for 5 minutes at 27° C. and then centrifuged at low speed. The solutions were assayed for lytic activity prior to and following suspension of the red cells. Assuming that the amount of kinase activity which disappeared from the liquid phase was adsorbed on the solid phase, the character of the curve in figure 2 indicates that the amount of urokinase adsorbed is directly proportional to the concentration of urokinase until the amount of bound urokinase reaches 80 centiunits/ml. in systems containing 10 percent erythrocytes by

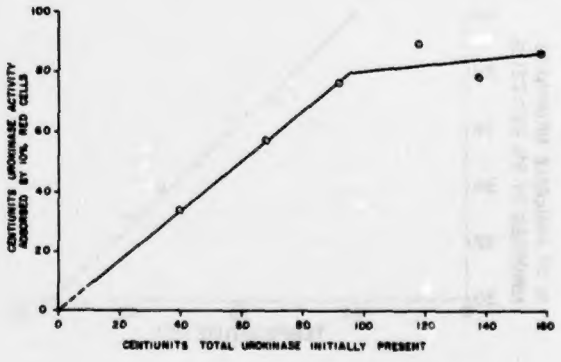


**FIGURE 2**

*Effect of variation in urokinase concentration on amount of urokinase absorbed by erythrocytes.*

volume. The presence of higher concentrations of urokinase than this does not materially increase the amount of urokinase adsorbed.

The effect of temperature on the adsorption phenomenon was also studied. One ml. suspensions were formed by mixing equal volumes of 6 percent sheep erythrocytes and veronal-saline buffer, pH 7.4, containing 75 centiunits of urokinase activity. These suspensions were incubated for 30 minutes at temperatures ranging from 20° C. to 47° C. and then centrifuged at low speed. The separated supernatant solutions were tested for remaining urokinase activity. For control purposes solutions containing 75 centiunits of urokinase in veronal-saline buffer were also assayed. The straight-line relationship shown in figure 3 indicates that the adsorption of urokinase by the concentration of red cells used is inversely proportional to the temperature of incubation.

Since plasma has been observed to be inhibitory to urokinase (Celander and Guest, unpublished observations) and since erythrocytes adsorb urokinase, experiments were performed to determine whether or not constituents inhibitory to urokinase could be adsorbed from plasma by washed erythrocytes. Whole blood obtained from healthy donor dogs was centrifuged at low speed and the plasma removed and stored. The buffy coat and upper 1 to 2 mm. of the red cells were removed and discarded. The remaining packed cells were suspended in an equal volume of veronal-saline buffer, pH 7.4, and then centrifuged at low speed. Sedimentation and resuspension were repeated 5 times. An aliquot of erythrocytes from each sedimentation procedure was retained. Five-tenths ml. aliquots of the washed red cells were suspended in 0.5 ml. aliquots of the original plasma. The reconstituted blood was incubated at 27° C. for 10 minutes and then centrifuged at low speed. The supernatant plasma from each of the 5 suspensions of washed red cells in plasma was analyzed for inhibitors to urokinase. The analysis was performed by adding a solution containing 350 centiunits of urokinase dissolved in 0.5 ml. of saline to a 0.5 ml. aliquot of the supernatant plasma. The mixtures were incubated at 27° C. for 10 minutes and tested for fibrinolytic activity. The curve plotted in figure 4 indicates that the inhibitory capacity of plasma was decreased by its exposure to washed erythrocytes and that the magnitude of the decrease bore a positive relationship to the number of times the red cells had been washed before being placed in the plasma.

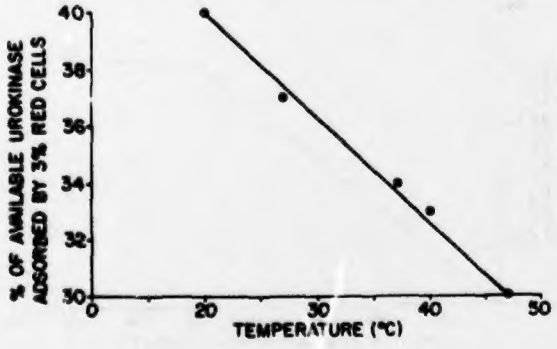


FIGURE 3

*Influence of temperature upon adsorption of urokinase by erythrocytes.*

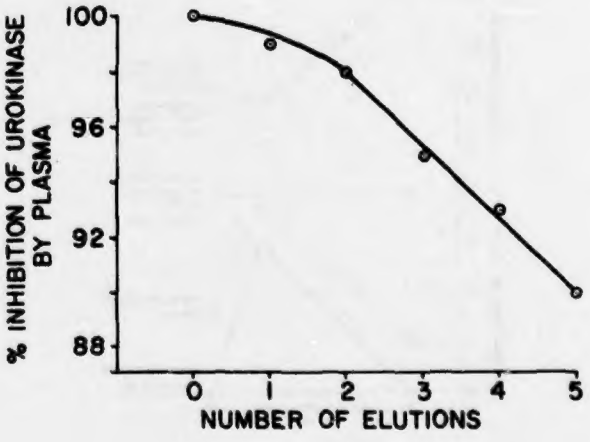


FIGURE 4

*Adsorption of plasma inhibitors by red cells after elution.*

To determine whether or not urokinase was still effective after adsorption on erythrocytes, packed canine erythrocytes were washed 3 times in 3 volumes of veronal-saline buffer, pH 7.4, and sedimented at low speed. The supernatant and the upper layer of the cells were discarded; the remaining red cells were suspended in an equal volume of veronal-saline buffer and 2 ml. of the suspension were mixed with 1 ml. of urokinase solution containing 350 centiunits of activity. After the mixture had been incubated at 27° C. for 10 minutes the cells were sedimented at low speed, washed once with an equal volume of veronal-saline buffer, and assayed for urokinase activity in two systems: one containing an equal volume of 0.9 percent saline; the other, an equal volume of plasma. In control experiments, 1 ml. of urokinase solution (350 centiunits) was added directly to an equal volume of 0.9 percent saline or plasma. The data are presented in table II. Most of the urokinase added directly to plasma disappeared, but when absorbed on red cells urokinase exhibited a greater lytic activity in plasma than it did in saline.

In a modification of the above experiment, 1 m'. of urokinase (350 centiunits) was added directly to 1 ml. of fresh whole blood (system A). Urokinase in the same volume and concentration was also added to 1 ml. of plasma. The erythrocytes which had been separated from this plasma were washed 3 times with 3 volumes of veronal-saline buffer and then recombined with the plasma (system B). System C consisted of 0.5 ml. of packed red cells which had been exposed to 350 centiunits of urokinase and washed as described previously before being suspended in 0.5 ml. of their native plasma. The results of analysis of the liquid phase of the 3 systems for the quantity of urokinase activity remaining are presented in table III. These data reveal that the most fibrinolytic of the 3 systems was that which contained washed red cells exposed to urokinase before resuspension in the plasma (system C).

Because fibrinolytic activity can be initiated in the plasma of man, dog, cat, and several other animals by streptokinase (13), an exotoxin produced by $\beta$ hemolytic streptococci, we investigated whether or not streptokinase-sensitive materials can also be adsorbed by erythrocytes. In preliminary experiments it was found that canine red cells, washed but otherwise untreated, contained a small amount of streptokinase-sensitive activity. This activity could not be removed completely by repeated washing, though it was reduced to a constant level. A preliminary report on the streptokinase-sensitive activity remaining on erythrocytes has been made (14).

The effect of activation of the fibrinolytic system in vivo on the ability of red cells to adsorb urokinase or plasma factors was investigated with dogs as the experimental subjects. In our investigation (results to be published) the intravenous infusion of diluted beef lung thromboplastin had been found to induce fibrinolytic activity in dogs. Whole blood

TABLE II

*Sensitivity of urokinase to plasma inhibitors*

| Source of kinase | Saline | | Plasma | | Activity in plasma relative to that in saline |
|---|---|---|---|---|---|
| | Mean* | S.E.$_m$ | Mean* | S.E.$_m$ | |
| Urokinase solution† | 833 | 40 | 45 | 12 | 95 percent reduction |
| Urokinase adsorbed on red cells‡ | 8 | 2 | 90 | 18 | Tenfold increase |

*The averages of 10 samples are expressed in terms of the mean reciprocal lysis time in hours × 100.

†Urokinase solution containing 350 centiunits was added to an equal volume of saline or plasma.

‡Urokinase-treated red cells were added to equal volumes of saline or plasma.

TABLE III

*Preservation of urokinase on washed erythrocytes*

| | Residual urokinase activity | |
|---|---|---|
| | Mean reciprocal lysis time in hours × 100§ | Standard error of the mean |
| Urokinase added to: | | |
| Saline* | 833 | 40 |
| Whole blood* (system A) | 22 | 2 |
| Whole blood* † (system B) | 46 | 5 |
| Whole blood‡ (system C) | 68 | 2 |
| Controls — saline added to: | | |
| Whole blood (system A) | 0 | — |
| Whole blood† (system B) | 0 | — |

*Urokinase added to each system was 350 centiunits, which is equivalent to 833 reciprocal lysis time units.

†Washed red cells were resuspended in their native plasma to form "whole blood."

‡Washed red cells were suspended in 350 centiunits of urokinase, washed once in veronal-saline buffer prior to their resuspension in native plasma.

§The averages of 10 samples are expressed in terms of the mean reciprocal lysis time in hours × 100.

from such animals as well as red cells and plasma were assayed for inhibitors to urokinase and the results compared with parallel assays of blood taken from the same animals prior to the induction of fibrinolysis. For a given experiment, 5 ml. of whole blood were withdrawn from the animal; 1 ml. of this was stored in the cold and the remainder was separated into plasma and erythrocytes. The buffy coat and the red cells of the top 1 to 2 mm. of packed cells were removed and discarded. The remaining cells were washed 3 times with veronal-saline buffer. Urokinase (0.5 ml.) containing 350 centiunits was mixed with one of the following: 0.5 ml. of whole blood, 0.5 ml. of a veronal-saline solution, 0.5 ml. of a solution containing an amount of plasma equivalent to that present in 0.5 ml. of whole blood, or with 0.5 ml. of the first red cell wash. Control solutions in which veronal-saline buffer was used in place of the urokinase solution were also assayed. The results are presented in table IV. Inhibition of urokinase by whole blood and plasma was decreased by the induction of mild fibrinolytic activity; however, the inhibitory activity of the wash from the red cells of fibrinolytic animals was increased. On the other hand, washed erythrocytes from 3 of the 10 fibrinolytic animals possessed fibrinolytic activity in the absence of urokinase. The eluates of these cells also contained fibrinolytic activity.

## DISCUSSION

The experiments, described above, indicate that erythrocytes possess, to a variable degree, the property of adsorbing factors involved in the fibrinolytic enzyme system. That the reaction is one of adsorption is evidenced by the fact that it is possible to recover the adsorbed agents (table I) as well as by an inverse relationship between the amount of material adsorbed and the temperature (fig. 3).

The type of behavior found in dog blood — that is, development of fibrinolytic activity accompanied by an increase in the concentration

TABLE IV

*Comparison of the blood components from fibrinolytic and nonfibrinolytic dogs*

| | Fibrinolytic activity | | | |
|---|---|---|---|---|
| | Prior to in vivo induction of fibrinolysis | | Following in vivo induction of fibrinolysis | |
| **Without added urokinase*** | Mean† | S.E.$_m$ | Mean† | S.E.$_m$ |
| Whole blood | 0 | — | 0 | — |
| Plasma | 0 | — | 0 | — |
| Red cell eluate | 12 | 4 | 12 | 4 |
| **With added urokinase‡** | | | | |
| Whole blood | 20 | 3 | 88 | 24 |
| Plasma | 37 | 5 | 430 | 50 |
| Red cell eluate | 333 | 60 | 200 | 30 |

*Volumes were corrected with saline.

†The averages of 10 samples are expressed in terms of the mean reciprocal lysis time in hours × 100.

‡In all instances 350 centiunits of urokinase were added (833 reciprocal lysis time units, S.E.$_m$ 40).

of inhibitors adsorbed to the surface of erythrocytes and a decrease in the amounts of inhibitors present in the plasma (table IV) — suggests that fibrinolytic activity develops when the erythrocytes remove a larger than normal amount of plasma inhibitors. It has been reported that, in the dog, there is normally present an activator (6) which is involved in the conversion of canine profibrinolysin to fibrinolysin. Since the tests for inhibitors herein described detect primarily inhibitors to kinase, it seems possible that depletion of such inhibitors by their adsorption to erythrocytes could alter the balance of the fibrinolytic system in such a manner as to permit plasma activators to bring about the conversion of profibrinolysin to fibrinolysin and hence result in the development of fibrinolytic activity. In this connection, it is significant that in addition to being fibrinolytic themselves, the red cells obtained from fibrinolytic animals yielded eluates which were at the same time fibrinolytic and inhibitory to urokinase (table IV). Thus not only were inhibitors to kinase removed from plasma by these erythrocytes, but fibrinolytic activity appeared on the erythrocytic surface.

Evidence is available that in man an increased concentration of erythrocytes, as seen in *essential polycythemia* vera (15), is accompanied by the development of mild fibrinolytic activity. It appears possible that an increase in the adsorption of one or more inhibitors to the activation of prokinase or in the adsorption of inhibitors to kinase by the increased number of erythrocytes is responsible for the development of the observed fibrinolytic activity. On the other hand, it is only fair to point out that secondary polycythemias occur in which fibrinolytic activity is not a salient feature.

The adsorption (table I) and apparent protection of urokinase (tables II, III) by erythrocytes suggest that these cells play a second role in the development of fibrinolytic activity. Active kinases generated at a local site might become concentrated through adsorption to erythrocytes present in the area, and subsequently bring about the activation of fibrinolysin with dissolution of the surrounding fibrin (table III). In vivo experiments are now in progress to ascertain the effectiveness of erythrocytes on which urokinase has been adsorbed as activators of the fibrinolytic system.

## REFERENCES


1. Ratnoff, O. D., and J. Colopy. Studies on a proteolytic enzyme in human plasma. IX. Fibrinogen and fibrin as substrates for the proteolytic enzyme of plasma. J. Clin. Invest. 32:473-479 (1953).

2. Bastian, J. W., and R. G. Hill. Factors in activation of plasminogen by streptokinase. Fed. Proc. 15:398 (1956).

3. Celander, D. R., and M. M. Guest. Factors influencing susceptibility of fibrin and fibrinogen to proteolysis by fibrinolysin. School of Aviation Medicine, USAF, Report No. 56-48, May 1956. Arch. Biochem. Biophys. 72:176-188 (1957).

4. Astrup, T. The biological significance of fibrinolysis. Lancet 2:565-568 (1956).

5. Johnson, S. A., and C. L. Schneider. The existence of antifibrinolysin activity in platelets. Science 117:229-230 (1953).

6. Lewis, J. H., and J. H. Ferguson. Studies on a proteolytic enzyme system of the blood. II. Fibrinolysokinase activators for profibrinolysin. J. Clin. Invest. 29:1059-1068 (1950).

7. Mohler, S. R., D. R. Celander, and M. M. Guest. Distribution of urokinase among the common mammals. School of Aviation Medicine, USAF, Report No. 57-147, Sept. 1957. Am. J. Physiol. 192:186-190 (1958).

8. Celander, D. R., and M. M. Guest. Purification and properties of a urinary activator of plasma profibrinolysin. Fed. Proc. 14:26 (1955).

9. Celander, D. R., R. P. Langlinais, and M. M. Guest. The application of foam technique to the partial purification of a urine activator of plasma profibrinolysin. Arch. Biochem. 55:286-287 (1955).

10. Ware, A. G., M. M. Guest, and W. H. Seegers. Fibrinogen: With special reference to its preparation and certain properties of the product. Arch. Biochem. 13:231-236 (1947).

11. Ware, A. G., and W. H. Seegers. Studies on prothrombin: Purification, inactivation with thrombin, and activation with thromboplastin and calcium. J. Biol. Chem. 175:565-575 (1948).

12. Guest, M. M. *Profibrinolysin, antifibrinolysin,* fibrinogen and urine fibrinolytic factors in the human subject. J. Clin. Invest. 33:1553-1559 (1954).

13. Guest, M. M., and D. R. Celander. Assay of human plasma prokinase. Am. J. Physiol. 187:602-603 (1956).

14. Celander, D. R., S. N. Kolmen, and M. M. Guest. Evidence for the presence of prokinase on human erythrocyte stroma. Abstracts of papers, 131st National Meeting, American Chemical Society, p. 43C, Apr. 1957.

15. Bjorkman, S. E., C. B. Laurell, and I. M. Nilsson. Serum proteins and fibrinolysis in polycythemia vera. Scandinav. J. Clin. & Lab. Invest. 8:304-308 (1956).